FEBS Letters 525 (2002) 126–130

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Rab3A and calmodulin regulate acrosomal exocytosis by mechanisms that do not require a direct interaction

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Received 21 June 2002; accepted 16 July 2002

First published online 25 July 2002

Edited by Felix Wieland

Abstract **The interaction between Rab3A and calmodulin is necessary for the inhibitory effect of Rab3A in neuroendocrine cells. Contrastingly, Rab3A triggers the exocytosis known as acrosome reaction in permeabilized spermatozoa. Here we show that a Rab3A mutant that cannot bind calmodulin was fully capable of triggering acrosomal exocytosis. Additionally, calmodulin by itself abrogated the exocytosis triggered by Rab3A. The effect was observed with both the wild type protein and the calmodulin binding deficient mutant. Our results indicate that the inhibitory and stimulatory effects of Rab3A in different exocytic processes are mediated by different effectors.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Rab3; Calmodulin; Acrosomal exocytosis; Permeabilized cell; Human spermatozoon

1. Introduction

The acrosome reaction is a specialized type of regulated exocytosis leading to a massive fusion between the outer acrosomal membrane and plasma membrane of sperm cells. Fusion between the two membranes occurs at multiple points, causing the release of the acrosomal content together with the external acrosomal membrane, the plasma membrane and part of the sperm cytoplasm [1]. We have previously described a model of streptolysin O permeabilized spermatozoa capable of undergoing acrosomal content release upon calcium stimulation which is suitable for unveiling some of the factors involved in this event [2,3].

Membrane fusion is governed by conserved families of proteins regardless of the trafficking pathway analyzed. The same basic mechanism is utilized for homotypic (e.g. fusion between early endosomes) and heterotypic (e.g. the exocytosis of synaptic vesicles) fusion. This conserved fusion machinery is present in cell types as diverse as yeast and mammalian neurons [4]. Rabs constitute a large family of small GTPases necessary for membrane fusion [5]. They recruit several Rab associated proteins responsible for the tethering of vesicles to the site where fusion is going to occur. Each Rab protein is associated with a specific intracellular compartment and participates in the membrane recognition and fusion events involving this compartment [6]. The more conspicuous Rab proteins found in secretory granules with a function in exocytosis are Rab3 isoforms. Although most studies report an inhibitory role for these proteins [7], a stimulatory role has been observed in some secretory events [8–11]. At present, it is difficult to reconcile the opposite effects of Rab3 in different secretion events [12]. Rab3A interacts with several putative effectors, such as RIM and rabphilin3A, although the mechanism by which these proteins mediate the biological effects of Rab3 is not well understood. Recent reports indicate that Rab3A effect on exocytosis is more likely related to interaction with RIM [13–15] than with rabphilin [16,17].

Rab3A is also a calmodulin binding protein [18–20]. Calmodulin is a calcium transducer protein that is expressed in all eukaryotic cells. By interacting with a number of different partners, calmodulin participates in signaling pathways that regulate many crucial processes such as growth, proliferation and movement. This protein has also been implicated in several exocytic events, including acrosomal exocytosis [21–23]. Because of its multiple functions, calmodulin is likely to regulate more than one step of the membrane fusion process. For example, recent evidence indicates that it participates at a late step of membrane fusion in association with proteolipids of V0, the membrane integral sector of the vacuolar H^+-ATPase [24,25]. Since both Rab3 and calmodulin participate in secretion, the interaction between these two proteins is likely relevant for exocytosis. In support of this idea, a Rab3A mutant that cannot bind calmodulin does not inhibit exocytosis when overexpressed in insulin and neurotransmitter secreting cells [19].

Acrosomal exocytosis is one of the few exocytic events where Rab3 has a distinct stimulatory role. In permeabilized spermatozoa, the process is inhibited by GDI, a protein that releases several Rab isoforms from membranes, and addition of recombinant Rab3A overcomes the inhibitory effect of GDI. Moreover, recombinant Rab3A loaded with the non-hydrolyzable nucleotide guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) triggers exocytosis [3,26]. The aim of the present work was to study whether the Rab3A–calmodulin interaction is important for triggering acrosomal exocytosis. The results indicate that this interaction can be disrupted without affecting Rab3A stimulatory function. Interestingly, calmodulin was found to inhibit exocytosis but by a mechanism not directly related to Rab3A binding.

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0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03102-2

2. Materials and methods

2.1. Reagents

Streptolysin O was obtained from Murex (Dartford, UK). Gamete Preparation Medium (GPM-Serono, Madrid, Spain) was used as culture medium. Botulinum neurotoxin C (BoNT/C, London strain) holotoxin was a kind gift from Ms. Laura de Jong and Dr. Rafael Fernández (Universidad Nacional de Cuyo, Mendoza, Argentina). The expression plasmid pGEX-2t containing the cDNA of bovine GDIα was generously provided by Dr. M.I. Colombo and Dr. P.D. Stahl (Washington University, St. Louis, MO, USA). Fluo3-AM was from Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma Chemical Company (St. Louis, MO, USA) or from ICN Biochemicals (Aurora, OH, USA). The Rab3A mutants used in this report have been described elsewhere [19]. The mutant Rab3A.V55 drastically reduces the interaction of Rab3A with RIM and rabphilin3A, while Rab3A.LT abolishes the binding to calmodulin without affecting the interaction with RIM and rabphilin3A.

2.2. Acrosome reaction in intact and permeabilized spermatozoa

Highly motile sperm from human healthy donors were recovered following a swim-up separation for 1 h in GPM medium at 37°C in an atmosphere of 5% CO_2, and concentration adjusted to $5–10\times10^6$/ml. Incubation proceeded for at least 2 h under conditions that support capacitation. For some experiments, spermatozoa without further treatment were used to study progesterone and A23187 induced acrosome reaction. For other experiments, sperm permeabilization was accomplished as described elsewhere [3]. Permeabilized sperm cells were resuspended in ice cold sucrose buffer (250 mM sucrose, 20 mM HEPES-K, 0.5 mM EGTA, pH 7) (SB) containing 2 mM dithiothreitol. Inhibitors were added when indicated and further incubated for 15 min at 37°C. After addition of stimulants, incubation proceeded at 37°C for 15 min. At least 200 cells were evaluated with fluorescein isothiocyanate–*Pisum sativum* according to Mendoza et al. [27]. The acrosome reaction was also evaluated in some instances by transmission electron microscopy, according to standard procedures [3].

2.3. Recombinant GDIα and Rab3A (wild type and mutants)

Recombinant GST fusion proteins were expressed in *Escherichia coli* and purified following standard procedures [3]. Rab3A (wild type and mutants) were prenylated in vitro as described [3]. Just before use, aliquots of the prenylated proteins were loaded with the non-hydrolyzable nucleotide guanosine GTPγS as described [3].

2.4. Fluo3-AM fluorescence

Non-permeabilized spermatozoa (10×10^6/ml) were incubated 30 min at 37°C in GPM in the presence of 10 μM of the membrane permeant Fluo3-AM. The cells were then washed with GPM at the same temperature. Aliquots of the sample were preincubated 15 min at 37°C with or without 25 μM of the calmodulin inhibitor W7. After transferring the samples to the cuvettes of an Aminco SLM 8000 spectrofluorometer equipped with a 450 W xenon arc lamp, 10 μM progesterone was added to both groups. Fluo3-AM fluorescence at 515 nm (emission) was registered at 30 s intervals during 10 min by exciting at 490 nm. At the end of the incubation, fluorescence in the presence of 10 μM A23187 was also measured.

2.5. Statistical analysis

Data were normalized by subtracting the number of reacted spermatozoa in the negative control (no stimulation) from all values. The resulting values were expressed as a percentage of the reacted cells observed in the positive control (calcium in permeabilized spermatozoa, and progesterone or A23187 in intact cells). Values express the mean ± S.E.M. of at least three independent experiments. Differences were tested by a two-way ANOVA and Fisher's PSLD tests. Percentages (not normalized) were transformed to the arc-sine prior to analysis. Only significant differences ($P<0.05$) among experimental groups are discussed.

3. Results

3.1. Binding of calmodulin to Rab3A is non-essential for Rab3A triggered exocytosis

Rab3A is a small GTPase that participates in exocytosis. This protein plays an inhibitory role in several secretory events. It has been shown that a mutant of Rab3A unable to bind calmodulin fails to inhibit exocytosis. In contrast, mutants that cannot bind other Rab3 effectors, such us RIM and rabphilin, are still capable of abrogating secretion [19]. These observations suggest that the inhibitory role of Rab3 is transduced through its interaction with calmodulin but not through other effectors.

In acrosomal exocytosis, Rab3 plays a stimulatory role. Therefore, it was interesting to test these mutants in the acrosomal exocytosis assay to evaluate whether the inhibitory and stimulatory effects of the protein in different exocytic events are mediated by the same effectors.

As we have previously reported, GTPγS alone and GTP bound wild type Rab3A triggered exocytosis in an extent similar to calcium in permeabilized sperm (Fig. 1). Rab3A.LT, a mutant defective in binding to calmodulin, was as effective as the wild type in promoting acrosomal exocytosis (Fig. 1). In contrast, Rab3.V55, a mutant that cannot interact with other effectors, could not trigger exocytosis (Fig. 1). When tested in combinations with other stimuli (calcium or GTPγS), Rab3A.V55 was not inhibitory, suggesting that the lack of activation could not be accounted for by a contaminant in the preparation. This observation also indicates that the mutant does not behave as a dominant negative factor in the assay.

The results indicate that – contrary to the situation in neuroendocrine cells – the interaction of Rab3A with calmodulin is not essential for triggering exocytosis, whereas interaction with other effectors is necessary.

3.2. Calmodulin regulates acrosomal exocytosis but not by direct interaction with Rab3A

Calmodulin has been reported to participate in several events of exocytosis. We tested the effect of purified calmodulin in acrosomal exocytosis. This protein inhibited calcium and Rab3A triggered exocytosis with a ED_{50} of 0.8 μM. Interestingly, exocytosis stimulated by Rab3A.LT, the mutant that does not bind calmodulin, was also inhibited by calmodulin (Fig. 2). These observations suggest that although calmodulin plays an important role in acrosomal exocytosis, it is not related to its binding to Rab3A.

3.3. Calmodulin inhibitors trigger acrosomal exocytosis in permeabilized spermatozoa

Since addition of exogenous calmodulin abrogates acrosomal exocytosis, we tested the effect of inactivating sperm endogenous calmodulin by using specific inhibitors. Consistent with a regulatory role for calmodulin, the inhibitors promoted exocytosis. The more potent inhibitors W7 and W13 triggered acrosomal secretion at 1 μM, whereas W5, a less potent calmodulin inhibitor, required 10 times higher concentrations to achieve the same effect (Fig. 3 and data not shown). Chlorpromazine, another calmodulin inhibitor chemically unrelated to W7 and W13, also triggered acrosomal exocytosis.

Two pieces of evidence indicate that the acrosomal exocytosis promoted by calmodulin inhibitors bears similarities with that initiated by calcium. First, the effect was sensitive

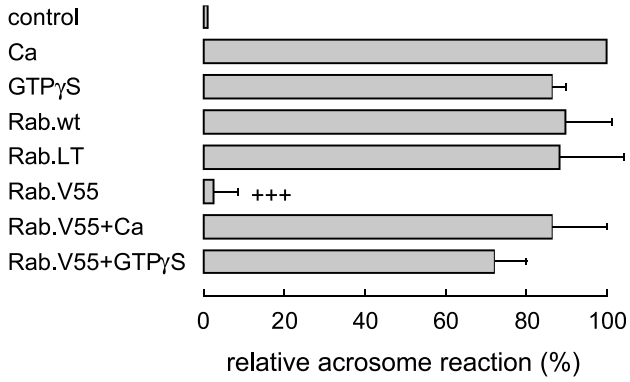

Fig. 1. Effect of Rab3A mutants on acrosomal exocytosis in permeabilized spermatozoa. Permeabilized spermatozoa were incubated for 15 min at 37°C in the absence of any stimulus (control), or in the presence of 10 μM free Ca^{2+} (Ca), 40 μM GTPγS (GTPγS), or 300 nM of the following proteins: Rab3A.wt (Rab.wt), Rab3A.LT (Rab.LT) or Rab3A.V55 (Rab.V55). To test the inhibitory effect of Rab3A.V55, 300 nM of the protein was added in the assay and incubated for 15 min at 37°C prior the addition of calcium or GTPγS. Ranges for negative and positive controls were 8–25% and 20–45%, respectively. Difference from Rab3A.wt statistically significant (***$P < 0.001$).

to GDI, a protein that sequesters Rabs from the membranes [28]. Second, activation by W7 was inhibited by BoNT/C, a toxin that cleaves the SNAREs syntaxin and SNAP-25. We have previously shown that calcium triggered acrosomal exocytosis requires Rab3A and SNAREs. Finally, to observe the morphological characteristics of the acrosomal exocytosis stimulated by W7, the sperm cells were fixed and observed by transmission electron microscopy. Addition of W7 increased the percentage of cells lacking the acrosome and exposing the inner acrosomal membrane without any noticeable morphological alterations as compared with calcium stimulated spermatozoa (data not shown).

3.4. *In intact spermatozoa, calmodulin inhibitors show opposite effects in progesterone and A23187 triggered acrosomal exocytosis*

In intact spermatozoa, several authors have reported that

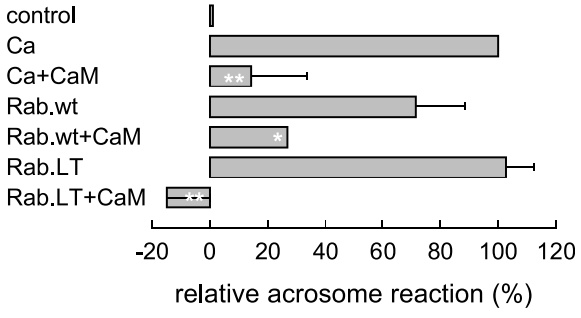

Fig. 2. Calmodulin inhibits acrosomal calcium and Rab3A triggered acrosomal exocytosis. Permeabilized spermatozoa were incubated for 15 min at 37°C in the presence or absence of 5 μM calmodulin (CaM). Afterwards, 10 μM free Ca^{2+} (Ca), or 300 nM of Rab3A.wt (Rab.wt) or Rab3A.LT (Rab.LT) was added to the system and the incubation was continued for an additional 15 min. The control aliquot did not receive any stimulation. Ranges for negative and positive controls were 13–25% and 26–35%, respectively. Difference from the corresponding condition without calmodulin statistically significant (*$P < 0.05$; **$P < 0.01$).

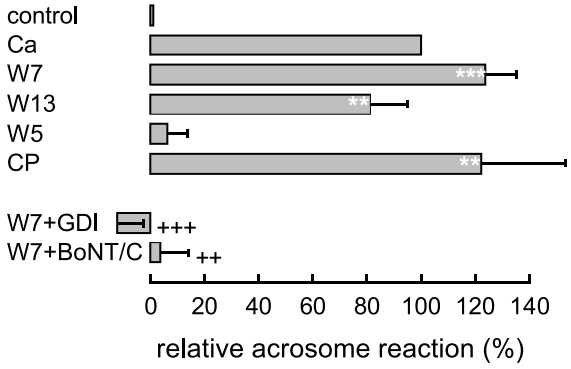

Fig. 3. Calmodulin inhibitors trigger acrosomal exocytosis. Permeabilized spermatozoa were incubated in the presence of 1 μM W7, W13 or W5, or 30μM chlorpromazine (CP) for 15 min at 37°C. In some experiments, the spermatozoa were preincubated for 15 min at 37°C with 4 μM GDI or 10 nM BoNT/C prior to the incubation with W7. Ranges for negative and positive controls were 14.5–27% and 26–46%, respectively. Difference from the control (no stimulus) statistically significant (**$P < 0.01$; ***$P < 0.001$). Difference from W7 statistically significant ($^{++}P < 0.01$; $^{+++}P < 0.001$).

calmodulin inhibitors abrogate acrosomal exocytosis initiated by physiological stimuli by interfering with calcium channels on the sperm surface [21–23]. These observations are in apparent contradiction with the stimulation we observed with the same inhibitors in permeabilized spermatozoa. Therefore, we assessed the effect of the inhibitors in intact spermatozoa that had undergone the same manipulations, except the permeabilization step. As shown in Fig. 4A, in intact spermatozoa, the calmodulin inhibitors W7, W13 and chlorpromazine were unable to initiate acrosome reaction. Moreover, they hampered exocytosis promoted by progesterone. W5, a less potent calmodulin inhibitor, was unable to trigger acrosome reaction or to prevent the effect of progesterone.

We assessed whether the cytosolic calcium increase triggered by progesterone was inhibited by calmodulin inhibitors. Progesterone caused a transient increase in cytosolic calcium early after addition. After a few minutes a second and sustained calcium increase was observed (Fig. 4B). Both calcium increases were inhibited by W7 (Fig. 4B,C). However, W7 did not interfere with the increase of fluorescence observed in the presence of a calcium ionophore, indicating that the calmodulin inhibitor did not interfere with Fluo3 fluorescence (Fig. 4C).

It has been shown that calmodulin inhibitors do not block acrosome reaction initiated by a calcium ionophore [21]. Results in Fig. 5A show that the inhibitors did not abrogate acrosome reaction elicited by A23187. On the contrary, a small but consistent stimulation was observed with W7 and chlorpromazine, suggesting that under appropriate conditions, the calmodulin inhibitors may facilitate acrosome reaction as they do in permeabilized spermatozoa. Therefore, we assessed the effect of the inhibitors at early times after the addition of the calcium ionophore, when most of the sperm cells have still not reacted. Fig. 5B shows that pretreatment with the calmodulin inhibitors strongly stimulate exocytosis 5 min after A23187 addition.

These results indicate that calmodulin regulates several steps of the acrosome exocytosis. The protein is necessary for the acrosome reaction initiated by physiological inducers that require the opening of calcium channels at the sperm

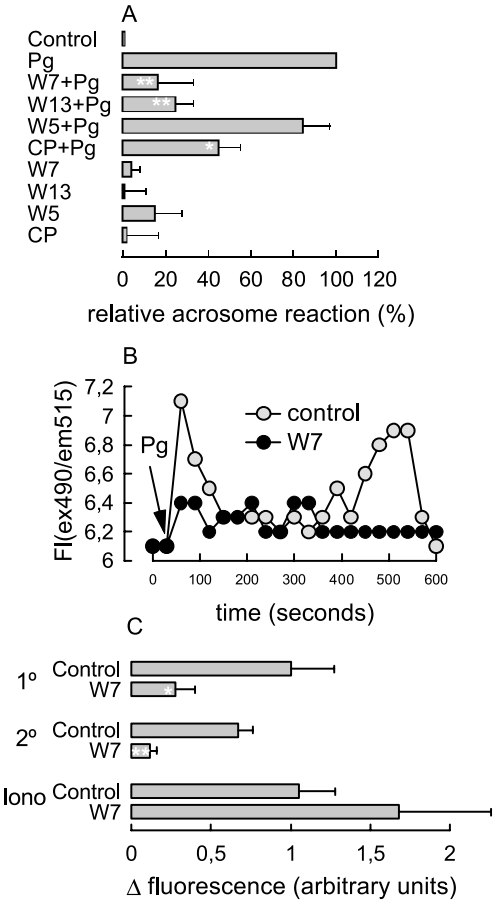

Fig. 4. Calmodulin inhibitors block acrosome reaction induced by progesterone by interfering with calcium channels. A: Non-permeabilized spermatozoa were preincubated for 15 min at 37°C in the absence or presence of 25 μM of W7, W13 or W5, or 30 μM chlorpromazine (CP). 10 μM progesterone was added to some samples for 15 min at 37°C. Ranges for negative and positive controls were 15–36% and 27–54%, respectively. Difference from progesterone statistically significant (*$P<0.05$; **$P<0.01$). B: Non-permeabilized spermatozoa were loaded with Fluo3-AM and preincubated 15 min at 37°C with (W7) or without (control) 25 μM W7. 10 μM progesterone was added to both groups (arrow). Fluorescence was recorded in a spectrofluorometer at 30 s intervals during 10 min (ex. 490 nm, em. 515 nm). Result of one representative experiment. C: Effect of W7 in the first and second fluorescence increase observed after progesterone treatment in four independent experiments performed as described in B. At the end of the incubation, 10 μM A23187 was added and the increase of fluorescence was measured in both groups (Iono). Difference between control and W7 statistically significant (*$P<0.05$; **$P<0.01$).

surface. However, when the cytosolic calcium increase is elicited by a mechanism that does not require these channels, calmodulin has a modulatory role that slows down exocytosis. As expected, in permeabilized cells, only the inhibitory role is observed.

4. Discussion

In most intracellular events of membrane fusion, specific Rab proteins must be activated to promote the aggregation of tethering proteins that hold together the membranes that are going to fuse [6]. This first attachment process is a required step for the tighter apposition of membranes mediated by trans SNARE complex assembly. However, a requirement for Rab3 has not been observed for the regulated exocytosis of neurotransmitters and hormones [7]. In many neuroendocrine cells, a fraction of the secretory vesicles are pre-docked at the plasma membrane and may have passed the tethering step. In this scenario, activation of Rabs may not be necessary for the exocytosis of this vesicle pool. The tethering of vesicles might be mediated by unidentified Rabs or by a Rab independent mechanism. Notwithstanding, it has been observed that in Rab3A knockout mice, the replenishment of docked vesicles after exhaustive stimulation is impaired suggesting that Rab3A may be necessary for the recruitment of new vesicles to the plasma membrane [29].

Although the presence of pre-docked vesicles or the existence of Rab3 independent tethering processes can explain a lack of a Rab3 requirement for exocytosis, these mechanisms cannot account for the inhibitory effect observed by the overexpression of Rab3 isoforms in several cell lines. Rab3 is special among other Rab proteins because it binds calmodulin [18]. Therefore, Rab3 may have a regulatory role in the membrane fusion process that is not present in other Rabs. This hypothesis has been directly assessed by generating a mutant of Rab3A that does not interact with calmodulin [19]. The fact that overexpression of this mutant is not inhibitory for exocytosis in insulin and neurotransmitter secreting cells strongly supports the idea that interaction with calmodulin is important for Rab3A inhibition of exocytosis. Consistent with this finding, association between Rab3A and calmodulin is inhibited by stimuli that promote exocytosis [20]. On the other hand, mutations that hinder the interaction of Rab3A with effectors such as rabphilin3A and RIM maintain their inhibitory effect on neuroendocrine cell secretion [19]. In contrast with what is observed in these cell types, Rab3A has a stimulatory effect in acrosomal exocytosis [3,26]. This feature renders the study of the acrosome reaction a powerful model

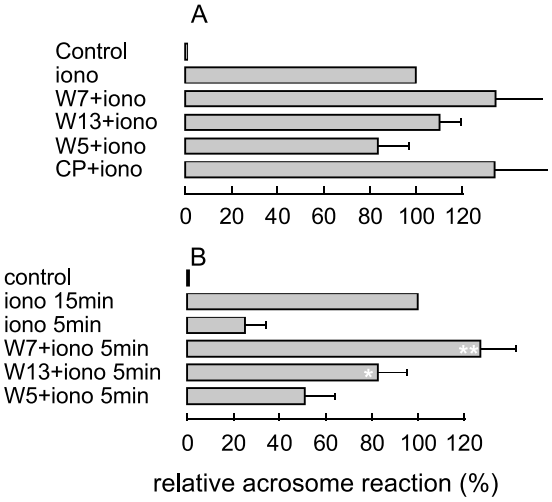

Fig. 5. Calmodulin inhibitors stimulate acrosome reaction induced by the ionophore A23187. Non-permeabilized spermatozoa were preincubated for 15 min at 37°C in the absence or presence of 25 μM of W7, W13 or W5, or 30 μM chlorpromazine (CP). 10 μM A23187 was added to the indicated samples. Acrosome reaction was evaluated after 15 min (A) or 5 min (B) at 37°C. Ranges for negative and positive controls were 7–36% and 36–61%, respectively. Difference from the ionophore (5 min) statistically significant (*$P<0.05$; **$P<0.01$).

where to apply all these mutants and thus test whether the inhibitory and activating effects of Rab3 are mediated by the same effectors. Our results clearly indicate that the two effects of the protein are mediated by different protein–protein interactions. Thus, binding to calmodulin is important to modulate exocytosis in neuroendocrine cells, whereas interaction with other effectors – such as RIM or rabphilin3A – is necessary to promote acrosomal exocytosis.

Interestingly, exogenous calmodulin showed a prominent inhibitory effect on acrosomal exocytosis in permeabilized spermatozoa. The effect was not directly related to interaction with Rab3A, since it inhibited equally well exocytosis promoted by wild type Rab3A or by the mutant that does not bind calmodulin. Park et al. [30] have recently reported that calmodulin may have a role in stimulating GTP binding to Rab3A. In our experiments, recombinant Rab3A was loaded with a non-hydrolyzable GTP analog, bypassing the activation step. Hence, the importance of calmodulin in Rab3a activation was not assessed.

Consistent with the hypothesis that calmodulin negatively modulates acrosomal exocytosis, inhibitors of the protein triggered exocytosis in permeabilized cells. It has been reported that, in intact cells, the same inhibitors abrogate acrosome reaction initiated by ZP [21,22]. We found similar results in progesterone induced acrosome reaction in non-permeabilized spermatozoa. These seemingly conflicting results can be explained if calmodulin participates at several steps in the acrosome reaction. In intact cells, calmodulin inhibitors abrogate the opening of calcium channels that is a relatively early step of the process (Fig. 4B,C and [21]). Both ZP3 and progesterone triggered exocytosis require a calcium flux through calcium channels in the plasma membrane [21,23,31]. Hence, it is expected that calmodulin inhibitors should prevent exocytosis by stimuli that require influx of calcium through channels. Other stimuli, such as addition of calcium ionophores, trigger acrosome exocytosis bypassing the requirement for calcium channel opening at the plasmalemma. Ionophore induced acrosome exocytosis was not inhibited by calmodulin inhibitors; on the contrary, these compounds strongly accelerated exocytosis mimicking the stimulatory effect observed in permeabilized sperm cells.

In summary, our results indicate that the inhibitory effect of Rab3A in neuroendocrine cells and the stimulatory effect in acrosomal exocytosis are not mediated by binding to the same effectors. The results also show that calmodulin has an active role at multiple steps of the acrosome reaction.

Acknowledgements: We thank M.I. Colombo for critical reading of the manuscript. This work was supported by an International Research Scholar Award from the Howard Hughes Medical Institute and by grants from CONICET (Argentina) and SECYT (Universidad Nacional de Cuyo).

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